

Sorghum Protein Extraction by Sonication and Its Relationship to Ethanol Fermentation

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ABSTRACT

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The objectives of this research were to develop a rapid method for extracting proteins from mashed and nonmashed sorghum meal using sonication (ultrasound), and to determine the relationships between the levels of extractable proteins and ethanol fermentation properties. Nine grain sorghum hybrids with a broad range of ethanol fermentation efficiencies were used. Proteins were extracted in an alkaline borate buffer using sonication and characterized and quantified by size-exclusion HPLC. A 30-sec sonication treatment extracted a lower level of proteins from nonmashed sorghum meal than extracting the proteins for 24 hr with buffer only (no sonication). However, more protein was extracted by sonication from the mashed samples than from the buffer-only 24-hr extraction. In addition, sonication extracted more polymeric proteins from

both the mashed and nonmashed samples compared with the buffer-only extraction method. Confocal laser-scanning microscopy images showed that the web-like protein microstructures were disrupted during sonication. The results showed that there were strong relationships between extractable proteins and fermentation parameters. Ethanol yield increased and conversion efficiency improved significantly as the amount of extractable proteins from sonication of mashed samples increased. The absolute amount of polymeric proteins extracted through sonication were also highly related to ethanol fermentation. Thus, the SE-HPLC area of proteins extracted from mashed sorghum using sonication could be used as an indicator for predicting fermentation quality of sorghum.

Sorghum (*Sorghum bicolor* L. Moench) is a drought-resistant and low-input cereal grain grown throughout the world, and interest in using it for bioindustrial applications is now growing in the United States (Farrell et al 2006). Although currently only $\approx 2.5\%$ of fuel ethanol is produced from grain sorghum, annual consumption of sorghum by the ethanol industry is steadily increasing from 11.25% in 2004 to 15% in 2005 and 26% in 2006 (Renewable Fuels Association 2005, 2006, 2007). Researchers and ethanol producers have shown that grain sorghum is a viable feedstock (technically acceptable, fits the infrastructure, and can be economically viable) for ethanol, and could make a larger contribution to the nation's fuel ethanol requirements.

Starch and protein are the two major components in sorghum grain. Recent research has shown that starch content is a good indicator of ethanol yield in the dry-grind process but starch content itself could not explain conversion efficiency well (Wu et al 2007). Sorghum varies in protein content from 6 to 18%, with 70–90% of the total protein belonging to the storage proteins (kafirins) (Lookhart et al 2000). According to previous research with 68 sorghum hybrids, a strong negative correlation was observed between ethanol yield and protein content ($R^2 = 0.60$, $P < 0.01$) (unpublished data), which is similar to data reported for soft wheat cultivars (Swanston et al 2007). However, multiple linear regression, including both starch and protein content as predictors, verified that protein content did not significantly contribute to ethanol yield ($P = 0.395$). The effect of protein content on conversion efficiency was statistically significant ($P = 0.015$) but represented only 8.6% of variation in efficiency (unpublished data).

Recently, we investigated the role of protein cross-linking to determine its impact on ethanol production. Protein digestibility, solubility, and microstructures were characterized for insight into

protein cross-linking occurring during the mashing process (Zhao et al 2008). Protein digestibility decreased significantly during mashing to levels lower than found in cooked sorghum foods. Likewise, protein solubility in an alkaline borate buffer containing SDS decreased substantially after mashing.

Confocal laser-scanning microscopy (CFLSM) images showed that web-like protein cross-links formed during mashing could trap oligosaccharides, polysaccharides, or starch and reduce the availability to enzymes during ethanol production (Wu et al 2007; Zhao et al 2008).

Therefore, protein cross-linking does have a significant effect on production of ethanol from sorghum (Zhao et al 2008). Protein digestibility, used as a marker of protein cross-linking of non-mashed sorghum meal, and protein solubility parameters showed positive correlation with conversion efficiency of sorghum. It is certain that most of the proteins soluble in the borate buffer would not be digested by yeast directly (Berry and Brown 1987). The role of protein solubility to predict ethanol fermentation could be related to protein structures which can determine the access of enzymes to native and gelatinized starch, polysaccharides, or oligosaccharides (Rooney and Pflugfelder 1986; Zhao et al 2008). The amount of total area under SE-HPLC had a better correlation with fermentation parameters than protein solubility and could be used as an indicator to predict ethanol-production quality of sorghum (Zhao et al 2008).

It is promising that the two indicators of degree of cross-linking occurring during mashing, protein digestibility and solubility, were highly correlated to fermentation parameters. To date, there is no rapid method that can be used to predict conversion efficiency of sorghum except for direct laboratory fermentation procedures. SE-HPLC has been widely used and provides automatic analysis, high accuracy, and utilizes only a small amount of sample. Therefore, SE-HPLC could be considered a good system for rapid characterization and quantization of proteins extracted from sorghum for predicting ethanol fermentation parameters. Sonication was used as a rapid method for extraction of unreduced wheat flour proteins by breaking down the glutenin fraction with the largest molecular size (Morel et al 2000; Singh and MacRitchie 2001; Singh et al 1990), presumably by the mechanical shear degradation (MacRitchie 1975; Singh et al 1990). Ultrasound has also been used to enhance the extraction of sorghum proteins (EI Nour et al 1998; Bean et al 2006). A method for isolating sorghum starch was also developed using sonication combined with buffers (Park et al 2006), that proved the effectiveness of sonication to separate starch from protein matrix rapidly.

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A quick method having a similar function as the 24-hr SDS borate buffer extraction method for sorghum protein extraction (Zhao et al 2008) is needed for predicting ethanol fermentation. Therefore, the combination of sonication with the extraction buffer and separation of proteins by SE-HPLC would make the extraction and analysis more efficient. Thus, the objective of this study was to develop a rapid method for sorghum protein extraction and analysis to predict ethanol fermentation.

MATERIALS AND METHODS

Sample Preparation

The nine sorghum hybrids (I–IX) described by Zhao et al (2008) were used and the methods for preparation of both mashed and nonmashed samples were unchanged in this study.

Extraction of Proteins Using Sonication

In total, 100 mg of nonmashed or mashed sorghum meal samples was mixed with 1 mL of 12.5 mM sodium borate, pH 10.0, containing 2% (w/v) SDS by vortexing for 10 sec in a 2-mL centrifuge tube to disperse the flour. The slurry was then sonicated with a Fisher F60 sonic dismembrator at an output of 10W for 30 sec. To prevent heat buildup, sample tubes were placed in an ice bath during sonication. After centrifugation at $13,200 \times g$ for 4 min, the supernatant was filtered through a 0.45- μ m membrane and analyzed directly by SE-HPLC. A separate 300 μ L of the filtrate was transferred by a pipette to a 2-mL vial and mixed with 6 μ L of β -mercaptoethanol (β -ME) to analyze the proteins under reducing conditions. For mashed samples, pellets were washed twice with the above borate buffer and once by water and then lyophilized, weighed, and analyzed for nitrogen content. Soluble nitrogen was calculated by subtraction of nitrogen in a freeze-dried pellet from total nitrogen in a mashed sample. Protein solubility was reported as the percentage of soluble nitrogen to total nitrogen.

Sequential Extraction of Proteins Using Sonication and Reducing Agent

In total, 100 mg of sample was first extracted using sonication as above. After centrifugation, the pellets were briefly washed twice with 12.5 mM sodium borate, pH 10.0, containing 2% (w/v) SDS and then extracted twice for 30 min for each extraction with 1 mL of 12.5 mM sodium borate, pH 10.0, containing 2% (w/v) SDS and 2% (v/v) β -ME. The supernatant from each extract was pooled 1:1 to produce the final extract for RP-HPLC.

Protein Characterization

SE-HPLC separation of extracted proteins was conducted using an Agilent 1100 HPLC system with a 300×7.8 mm BioSep-SEC-S3000 column (Phenomenex, Torrance, CA). The mobile phase was a pH 7.0 sodium phosphate buffer (50 mM) with 1% SDS (w/v) added. Flow rate was 1 mL/min with 15 μ L injection volume and column temperature was maintained at 40°C. Standard proteins, including thyroglobulin (669 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used to estimate M_w distribution of sorghum proteins separated by SE-HPLC. RP-HPLC of reduced proteins was conducted using an Agilent 1100 HPLC system equipped with a Jupiter C18 2.0×150 mm column (Phenomenex) with guard columns of the same material. Samples (10 μ L each) were injected and separated with a continuous linear gradient of 0.1% tri-fluoroacetic acid (TFA) (solvent A) and acetonitrile containing 0.1% TFA (solvent B), in which solvent B increased from 28 to 60.5% over 50 min and then was held 10 min (Bean et al 2000). Flow rate was 0.5 mL/min with column temperature maintained at 50°C. Proteins were detected by measuring UV absorbance at 214 nm. Peak areas were expressed in arbitrary units based on millivolts of detector output.

CFLSM Images

Mashed sample (100 mg) was first washed with 1 mL of H₂O three times. Proteins in a residual pellet were labeled by mixing with 1 mL of 0.05% (w/v) fluorescein isothiocyanate (FITC) solution (in 0.5 mM NaOH) and incubating it in the dark for 1 hr at room temperature. After centrifugation at $13,200 \times g$ for 4 min, the pellet was spread on a glass slide and dried at room temperature in the dark. Protein microstructure was visualized using a laser-scanning confocal microscope (Zeiss LSM 5 PASCAL; Carl Zeiss MicroImaging, Thornwood, NY). Before imaging, one drop of optical liquid immersion oil was added to the sample. A coverslip was placed on it, and another drop of oil was added on top of the coverslip to achieve higher resolution (Lee et al 2001). Sorghum protein fluorescence was analyzed using 488-nm excitation then through a 505–530 band-pass barrier filter for detection of FITC. Optical sections of samples were collected with a z -step of 0.9 μ m throughout the sample thickness. Three-dimensional images included >25 laser-generated optical planes in z -sectioning. Only one plane in the middle of the z -series is presented.

Nitrogen Quantitation

Nitrogen content of nonmashed and mashed samples and pellets after sonication extraction was analyzed by combustion according to Approved Method 46-30 (AACC International 2000) using a nitrogen determinator (model FP-528, Leco Corp., St. Joseph, MI). Nitrogen values were multiplied by 6.25 to convert to protein values.

Statistical Analyses

All experiments were performed at least in duplicate. Results presented are the mean values of the repeated experiments. Analysis of variance, least significant difference, and linear regression were performed using SAS software (v.9.1, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Sonication Extraction and Protein Solubility

By using the method of Wallace et al (1990), 91.3–95.7% of the total protein in whole sorghum grain was extracted using 12.5 mM borate buffer, pH 10.0, containing 1% SDS and 2% β -ME with extraction time of 1 hr (Hamaker et al 1995). When the detergent for extracting sorghum proteins was optimized to be 2% SDS at pH 10.0, the amount of extracted protein was similar compared with the control methodology but with a 35–80% extraction time reduction (Park and Bean 2003). For mashed sorghum, <5% of the total protein could be extracted using the above optimum buffer with two 30-min extractions under reducing conditions (Zhao et al 2008). Without using reducing agent, 77.2–92.6% of the total protein was extracted from nonmashed sorghum with the borate buffer when extracted for 24 hr but only 7.8–31.1% of the total protein was extracted from mashed sorghum (Zhao et al 2008).

Previous research indicated that the power setting and sonication time are the two main variables affecting protein extractability and size distribution when using ultrasound (Singh et al 1990; Morel et al 2000; Singh and MacRitchie 2001; Bean et al 2006). In other words, protein extraction rate is a function of ultrasonic energy (sonication time \times power) (Morel et al 2000). For example, most of proteins from strong as well as weak wheat flours can be extracted using a 30-sec sonication at power setting 5 (an output of 10W) (Singh et al 1990; Singh and MacRitchie 2001), and the solid-to-solvent ratio of 1:10–1:90 did not significantly affect the extractability when enough ultrasonic energy was delivered to the flour sample (Singh et al 1990). However, oversonication could cause excessive depolymerization of glutenin (Singh et al 1990; Morel et al 2000). Our research showed that the optimum combination of power output and sonication time for

extracting sorghum proteins is 10W for 30 sec, in which the maximum level of proteins can be extracted from original sorghum without oversonication (unpublished data).

Both solubility and SE-HPLC peak area of proteins extracted from mashed sorghum using the borate buffer with extraction time of 24 hr were highly correlated to ethanol fermentation (Zhao et al 2008). Ethanol yield increased and conversion efficiency improved notably with the increase of extracted proteins from mashed sorghum. After the encouraging results, we reproduced these effects using sonication in conjunction with the SDS borate buffer. By comparison with total area under the SE-HPLC chromatograms, the 30-sec sonication extracted fewer proteins from nonmashed sorghum than the 24-hr extraction with SDS borate buffer. The total area of proteins extracted using sonication accounted for 68.4–93.7% of the total area of those proteins extracted with the SDS borate buffer using a 24-hr extraction period (79.2% on average) (data not shown). Given that 77.2–92.6% of the total protein was extracted from nonmashed sor-

ghum with the SDS borate buffer and a 24-hr extraction (Zhao et al 2008), the 30-sec sonication could extract 52.8–86.5% of the total protein from the nonmashed sorghum (calculated by product of protein solubility with SDS borate buffer and the percentage of total area by sonication to that by the SDS borate buffer).

SE-HPLC total peak area from sonication extraction decreased substantially after mashing ($P < 0.0001$) (Fig. 1). Fewer proteins were extracted from mashed samples by sonication than the original counterparts, which coincides with the result that mashing reduced protein digestibility and solubility due to protein cross-linking (Zhao et al 2008). There was no significant difference in SE-HPLC total area between proteins extracted with SDS borate buffer for 24 hr and sonication extraction from mashed samples ($P = 0.08$). Protein solubilities using sonication were 12.0–31.1% with an average of 24.0%, slightly higher than those using the 24-hr SDS borate buffer extraction ($P < 0.0001$) (data not shown).

Protein Characterization by SE-HPLC

Typical SE-HPLC patterns of proteins extracted using sonication are shown in Fig. 2. For comparative purposes, the corresponding chromatograms of proteins using the 24-hr SDS borate buffer extraction in the previous study (Zhao et al 2008) are also displayed in Figs. 2 and 3. SE-HPLC chromatograms were divided into four regions (fractions I, II, III, and IV) (Zhao et al 2008). Based on the elution times of standard proteins, fraction I was considered as proteins with $M_w > 669$ kDa. It is noteworthy that all nonmashed sorghum samples had fraction I, with M_w much larger than individual kafirins (≈ 20 –30 kDa). This result confirmed the fact that a few large polymeric proteins already existed in nonmashed sorghum and were solubilized in some solvents with or without sonication (EI Nour et al 1998; Bean et al 2006; Zhao et al 2008).

For nonmashed sorghum, the proteins extracted with the borate buffer had larger total area than those extracted using sonication.

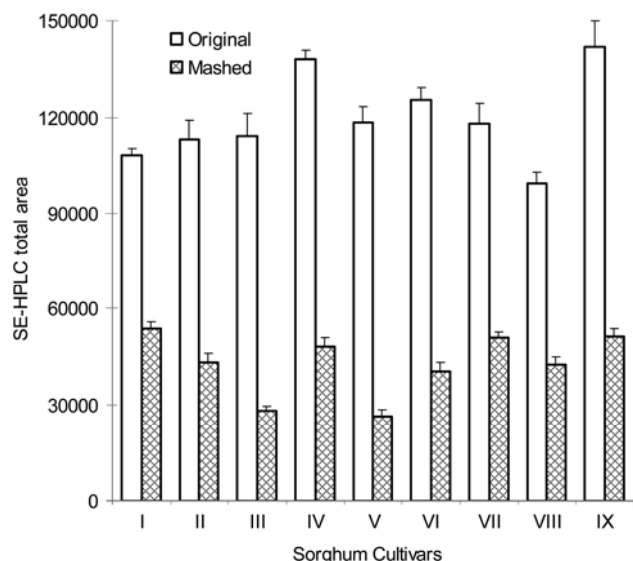


Fig. 1. Comparison of size-exclusion (SE)-HPLC total area of proteins extracted from original (nonmashed) and mashed sorghum using sonication. Bars represent standard deviations with four replications. Pooled standard errors were 3,217 for nonmashed samples and 1,694 for mashed samples.

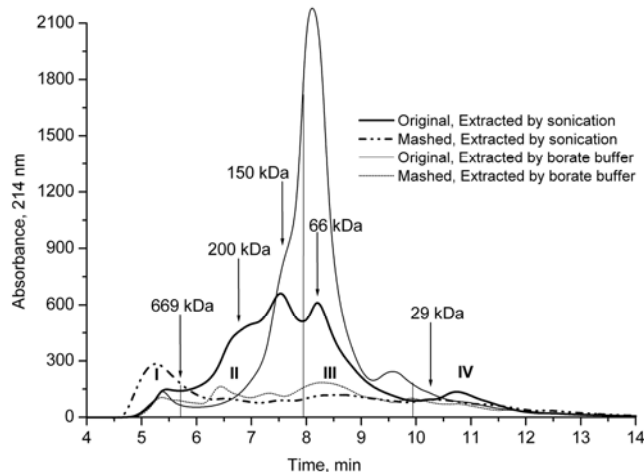


Fig. 2. Comparison of size-exclusion HPLC separations of proteins extracted from original (nonmashed) and mashed sorghum by sonication in a buffer (12.5 mM sodium borate, pH 10.0, containing 2% SDS) for 30 sec or only with the buffer for 24 hr.

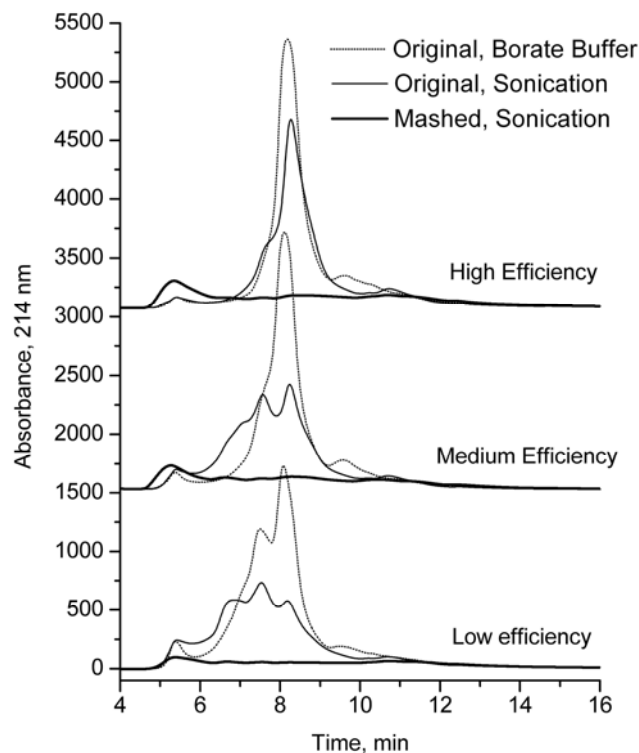


Fig. 3. Comparison of size-exclusion HPLC separations of proteins extracted from original (nonmashed) and mashed sorghum with different conversion efficiency by sonication in a buffer (12.5 mM sodium borate, pH 10.0, containing 2% SDS) for 30 sec or only with the buffer for 24 hr.

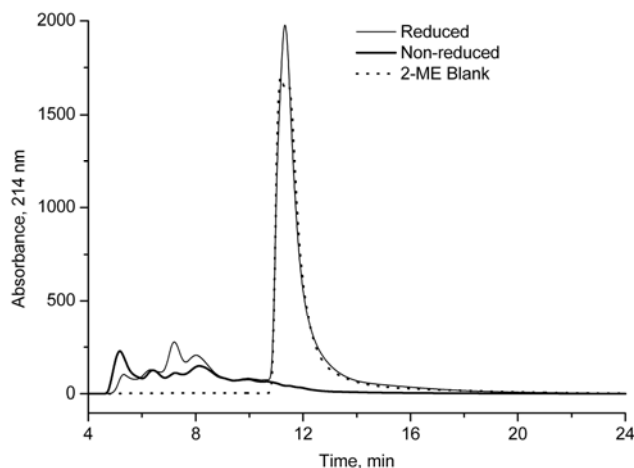


Fig. 4. Size-exclusion HPLC separations of proteins (extracted from mashed sorghum by sonication) before and after reduction by 2% (v/v) β -mercaptoethanol (β -ME).

Sonication tended to extract more polymeric proteins than borate buffer, which is clearly evident from Figs. 2 and 3. The chromatogram using sonication had a larger fraction I area as well as greater ratio of fraction II. Chromatogram of proteins extracted with borate buffer was much sharper, with a majority of the proteins eluting at ≈ 8 min. In contrast, chromatogram obtained using sonication appeared flattened with diffused peaks, and more proteins were eluted before 8 min. This flattening trend became more obvious for samples with lower conversion efficiency in Fig. 3. The increase in protein components eluted in fractions I and II using sonication extraction could be ascribed to the shear degradation of polymeric proteins that cannot be achieved by using the borate buffer alone. Sonication is believed to reduce the molecular weight of protein complexes by breaking covalent bonds, thus rendering them soluble (MacRitchie 1975; Singh et al 1990). Presumably, sonication extracted more polymeric proteins from the nonmashed samples with poorer conversion efficiency due to higher degree of protein cross-linking in those samples, as indicated by the lower protein digestibility in the previous study (Zhao et al 2008). It should also be pointed out that insolubility of proteins could result from factors other than simply M_w . For example, the proteins not easily extracted in buffer alone could be strongly aggregated through hydrophobic or other bonds. Changes to protein structure such as increases in β sheet may impede access of solvent to aggregated proteins (Byaruhanga et al 2006), reducing their solubility.

For mashed sorghum, the difference in SE-HPLC total area of proteins extracted by both methods was not significant, as mentioned above. The proteins extracted through sonication had more peak area for fraction I than those extracted with the SDS borate buffer alone, while the latter had higher proportions of fractions II and III. The chromatogram from mashed sorghum extracted using sonication slightly skewed left in comparison with the other three curves, indicating a broader M_w range (Fig. 2). The protein solubility of the mashed sample with low conversion efficiency in Fig. 3 was only 12.0%, which was much less than its counterparts (30.1 and 22.8% for the samples with high and medium conversion efficiency, respectively). Moreover, the M_w range of the proteins from this mashed sample became narrower in comparison with the other two samples with higher efficiency in Fig. 3. The variation in protein extractability of mashed sorghum under sonication corresponds to the differences in the ease of degradation of different polymers (Singh and MacRitchie 2001).

The 30-sec sonication extracted more cross-linked polymeric proteins from mashed sorghum than the 24-hr SDS borate buffer extraction. However, because only 12.0–31.1% of the total protein

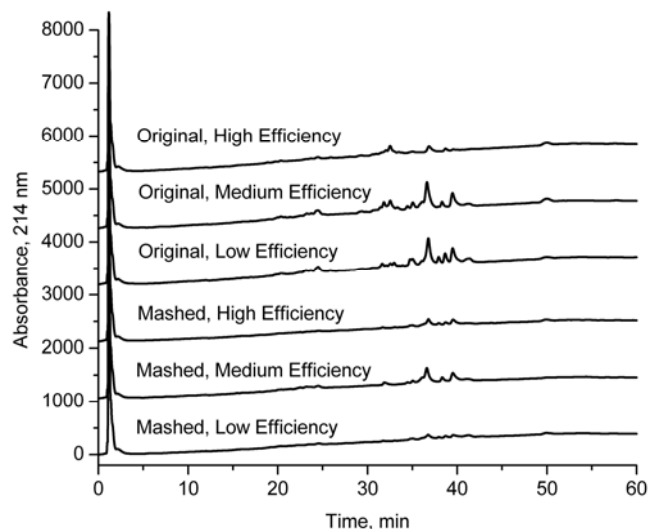


Fig. 5. Reversed-phase HPLC separations of proteins extracted using 12.5 mM, pH 10.0 sodium borate containing 2% SDS and 2% β -mercaptoethanol (β -ME) with two extractions of 30 min each from original (nonmashed) and mashed sorghum with different conversion efficiency.

in mashed sorghum was soluble with sonication extraction, fraction I accounted for only 1.7–6.3% of the total protein (calculated by the product of solubility and area ratio of fraction I).

For proteins extracted from mashed sorghum using sonication and separated by SE-HPLC (Fig. 4), the area of fraction I decreased significantly after reduction by 2% β -ME. It is obvious that high M_w polypeptides decreased while low M_w polypeptides increased, thus indicating that some of the proteins were linked by disulfide bonds. This is consistent with a recent report in which the polymeric proteins extracted from nonmashed sorghum and analyzed under nonreducing conditions by SE-HPLC were found to be disulfide linked together (Bean et al 2006) and have been reported in studies on cooked sorghum foods (Hamaker et al 1986, 1987; Duodu et al 2002; Ezeogu et al 2005). However, fraction I did not disappear completely after adding β -ME, indicating that some proteins could not be degraded by this reducing agent. Those protein molecules that still appeared at fraction I were taken as nondisulfide cross-linked polymers or, at minimum, resistant to reducing agents.

Sequential Extraction Using Sonication and Reducing Agent, and RP-HPLC Measurement

Borate buffer (12.5 mM, pH 10.0) containing 2% SDS and 2% β -ME was used to extract the residual protein in pellets after sonication. It was expected that 90–95% of the total protein in nonmashed sorghum could be dissolved in this solvent with two extractions of 30 min each (Hamaker et al 1995; Park and Bean 2003; Nunes et al 2004). RP-HPLC chromatograms in Fig. 5 show that the 30-sec sonication failed to dissolve all proteins in nonmashed sorghum and more proteins were further extracted under reducing conditions. Figure 5 also confirms that sonication preextracted more proteins from nonmashed sorghum with high efficiency than those with lower efficiency and thus fewer proteins were extracted with β -ME and detected by RP-HPLC.

In the previous study (Zhao et al 2008), β -ME further extracted 29.0–53.9% (44.0% on average) of the total protein from mashed residual sorghum pellets after preextraction with the SDS borate buffer for 24 hr, which demonstrated that a large amount of polymers cross-linked by disulfide bonds developed during mashing. There was not much difference in RP-HPLC patterns between proteins extracted by β -ME from residual pellets either after sonication or after preextraction with borate buffer for 24 hr. However, the total area under an RP-HPLC chromatogram of

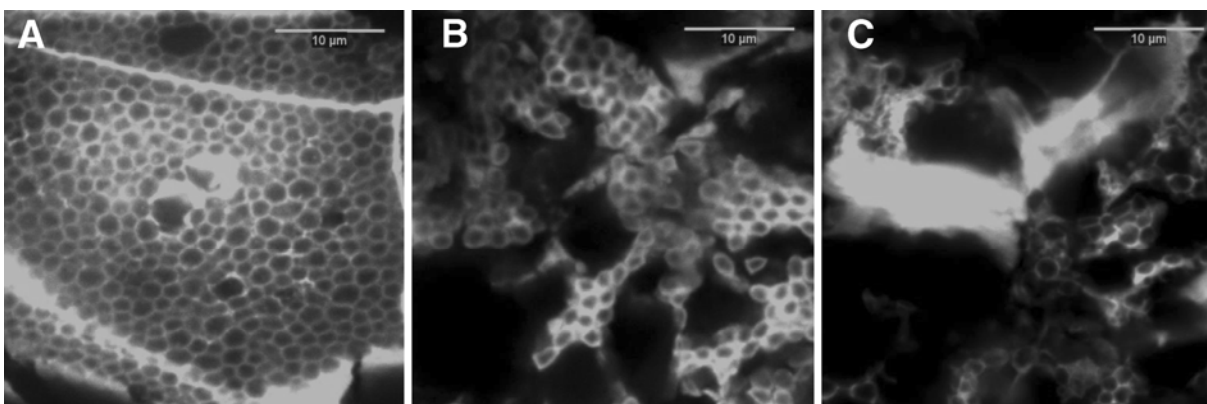


Fig. 6. Confocal laser-scanning microscopy images (single optical planes) of a sorghum cultivar with low-conversion-efficiency protein matrix (white-gray areas) stained with fluorescein isothiocyanate. **A**, Pellet after mashing; **B**, pellet after sonication extraction; **C**, pellet after sequential extraction using sonication and β -mercaptoethanol.

TABLE I
Coefficient of Determination (R^2) for Size-Exclusion (SE) HPLC Area of Proteins Extracted from Nine Sorghum Hybrids Using Sonication and Ethanol Fermentation^a

Ethanol Fermentation	Nonmashed Sorghum Total Area/mg of Protein	Mashed Sorghum		
		Total Area/mg of Protein	Area of Fraction I/ mg of Protein	Area of Fraction I/ mg of Protein, Reduced ^b
Ethanol yield ^c	0.32ns	0.83***	0.67**	0.83***
Conversion efficiency ^d	0.41ns	0.75**	0.65**	0.83***

^a SE-HPLC area calculated by dividing integrated area by mass of total protein in a sample. NS, not significant at 5% level; *, **, and ***, significant at 5, 1, and 0.1% level, respectively (from duplicate measurements).

^b Proteins reduced by 2% β -mercaptoethanol and fractionated by SE-HPLC after sonication extraction.

^c Amount of ethanol produced (v/v).

^d Calculated from theoretical yield of 56.79 g of ethanol produced from 100 g of dry starch.

proteins extracted after sonication was lower than extracted after the 24-hr SDS buffer extraction for all mashed samples (data not shown). This indicates that fewer proteins were extracted by β -ME from the residual pellets after preextraction by sonication. Although the solubility of proteins extracted using sonication was slightly higher than that extracted by the SDS borate buffer for 24 hr, it could not compensate for the difference in the area of RP-HPLC chromatograms. It is likely that the cross-linking in sections of protein matrix broken by sonication was intact enough to reduce access to remaining disulfide bonds by reducing agents. Zhao et al (2008) found that preextraction with the SDS borate buffer for 24 hr did help β -ME to extract more polymers cross-linked by disulfide bonds than direct extraction with β -ME.

Even sequential extraction using sonication or the borate buffer, and β -ME could not completely solubilize proteins in mashed sorghum. This result contrasts with a recent report in which the protein in cooked sorghum was completely dissolved in solvent with 12.5 mM borate (pH 10.0), 2% (w/v) SDS, and 1% (v/v) β -ME (Nunes et al 2004).

CFLSM Images and Protein Microstructures

CFLSM images proved that sorghum proteins tended to form highly extended, strong, web-like microstructures during mashing (Wu et al 2007; Zhao et al 2008). More open microstructures were observed in hybrids with higher conversion efficiencies (Zhao et al 2008). The changes of protein microstructure in a mashed sample during sequential extraction are shown in Fig. 6. The sample selected had the most obvious web-like protein matrix after mashing (Fig. 6A). Sections of this matrix were still visible after sonication (Fig. 6B). This was different from most samples in that, after sonication, only very small pieces of the web-like structures were still visible in the other samples. Figure 6C shows the web-like protein matrix after extraction of the residue with reducing agent, in which the matrix was further degraded. We also

observed that the intensity of white-gray areas that represented the quantity of protein matrix became weaker during sequential extraction using sonication and β -ME, indicating that more and more proteins were solubilized. CFLSM images confirm that the protein in mashed sorghum was not completely extracted.

Usefulness of SE-HPLC Profiles for Predicting Ethanol Fermentation

As reported by Zhao et al (2008), the nine sorghum hybrids used in this study had ethanol yields of 12.36–14.41% (v/v) (13.33% on average) and conversion efficiencies of 83.9–91.1% (87.1% on average). The coefficients of determination (R^2) for SE-HPLC area of proteins extracted from those hybrids using sonication and ethanol fermentation are listed in Table I. To make data from different samples with different protein contents comparable, area per milligram of protein was calculated by dividing the SE-HPLC total area or the area of fraction I by the mass of total protein (a product of protein content and mass of the sample used for extraction) in a sample. SE-HPLC total area of proteins from nonmashed sorghum did not correlate significantly to ethanol yield ($P = 0.113$) or conversion efficiency ($P = 0.062$). However, SE-HPLC total area of proteins from mashed sorghum strongly correlated with fermentation parameters ($R^2 = 0.83$, $P = 0.0007$ for ethanol yield and $R^2 = 0.75$, $P = 0.0025$ for conversion efficiency). Ethanol yield increased and conversion efficiency improved notably with increase in the amount of proteins extracted using sonication (i.e., these factors were highly correlated). The 30-sec sonication disrupted the highly cross-linked proteins in mashed sorghum and greatly enhanced solubility. Therefore, sonication in combination with SE-HPLC made the protein extraction and quantization process more efficient in terms of precision and time. SE-HPLC total area of proteins extracted from mashed sorghum using sonication could be used as an indicator for predicting fermentation quality of sorghum.

Absolute amount of polymeric proteins (fraction I) extracted using sonication was highly related to ethanol fermentation ($R^2 = 0.67$, $P = 0.007$ for yield and $R^2 = 0.65$, $P = 0.009$ for efficiency). After being reduced, the relationship between area of fraction I and fermentation parameters became stronger ($R^2 = 0.83$, $P < 0.0001$ for yield and $R^2 = 0.83$, $P = 0.0007$ for efficiency). SE-HPLC area of extractable nondisulfide cross-linked polymers from mashed sorghum using sonication could also be used as another indicator for fermentation performance.

In conclusion, the amount of proteins extracted using sonication and quantified by SE-HPLC indirectly reflected protein structures that can determine the access of enzymes to sorghum starch and thus could be related to fermentation quality of sorghum.

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